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# What should we call the Levant mole? Unravelling the systematics and demography of *Talpa levantis* Thomas, 1906 sensu lato (Mammalia: Talpidae).

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## Abstract

Turkey hosts five of the eleven species of *Talpa* described to date, Anatolia in particular appearing to be an important centre of diversity for this genus. Of these taxa, the Levant mole, *Talpa levantis* Thomas, 1906 has been suggested to consist of two genetically divergent sublineages, which may represent separate species. Here we use a combination of mitochondrial and nuclear DNA sequences from specimens of *T. levantis* s.lat. collected across the species geographical range to explore the systematics and demographic history of Levant moles. Both mitochondrial and nuclear markers confirm the existence of distinct eastern and western sublineages, which apparently diverged from each other in the early Pleistocene. Given the degree of cytochrome-*b* divergence between these (7.28%), we consider them to represent independent, cryptic species. By including topotypic specimens of *T. levantis* s. str. in our study we are able to show that this name applies to the western sublineage, distributed across most of the Anatolian Black Sea coastal region, from the vicinity of Trabzon in the east, westwards to Marmara. The earliest name available for the eastern taxon, found in Transcaucasia and adjacent parts of northeastern Anatolia, is *T. transcaucasica* Dahl, 1944. Cytochrome-*b* haplotype diversity in *T. levantis* is relatively high, demographic analyses suggesting that the species may have survived in multiple, separate, refugial areas during the Pleistocene. Our work brings the total number of named mole species recognized in Turkey to six, emphasising the importance of this region as a global centre of mole diversification.



**Key words:** *Talpa*; Cytochrome *b*; *BRCA2*; Phylogeny; Anatolia

## Introduction

*Talpa* Linnaeus, 1758 is a strictly subterranean genus of moles, distributed throughout the western Palearctic region, from the Iberian Peninsula to China and Siberia (Hutterer, 2005), which has been recovered as monophyletic in a number of phylogenetic studies (e.g., Colangelo et al., 2010; Bannikova et al., 2015). Bannikova et al. (2015) dated the most recent common ancestor of *Talpa* to 5.49–7.64 Myr, based on four concatenated nuclear genes, a time window in keeping with that suggested by Colangelo et al. (2010) from studies of mitochondrial cytochrome *b* (cyt-*b*) sequences, and corresponding to the Late Miocene (latest Tortonian and Messinian stages). During this time several dramatic changes to terrestrial environments and ecosystems occurred, large areas of continents experiencing drying, enhanced seasonality, and a consequent restructuring of terrestrial plant and animal communities (Herbert et al., 2016). The most recent version of *Mammal Species of the World* (Hutterer, 2005) recognized nine valid species in the genus, divided into a western group including the common mole *T. europaea* Linnaeus, 1758, the blind mole *T. caeca* Savi, 1822, the Roman mole *T. romana* Thomas, 1902, the Levant mole *T. levantis* Thomas, 1906, the Iberian blind mole *T. occidentalis* Cabrera, 1907 and the Balkan mole *T. stankovici* Martino and Martino, 1931 and an eastern group comprised of the Siberian mole *T. altaica* Nikolasky, 1883, the Père David's mole *T. davidiana* Milne-Edwards, 1884 and the Caucasian mole *T. caucasica* Satunin, 1908. Based on genetic data, Bannikova et al. (2015) recognized three additional species: *T. talyschensis* Vereschagin, 1945; *T. ognevi* Stroganov, 1948 and *Talpa* ex gr. *levantis*. More recently two new mole species, *T. aquitania* Nicolas, Martínez-Vargas and Hugot, 2017 (Nicolas et al., 2017a) from southern France and northern Spain and *T. martinorum* Kryštufek, Nedyalkov, Astrin and Hutterer, 2018 from the south-western Black Sea coast (Thrace), were described, initially on the basis of genetic data(Nicolas et al. (2017b; Kryštufek et al. 2018). While most species of *Talpa* are narrowly endemic with predominantly non-overlapping ranges, one species, *T. europaea,* is widespread and relatively eurytopic across much of Europe, with a range extending from the Ebro River in Spain to the Ob and Irtish Rivers in Russia (Mitchell-Jones et al., 1999; Hutterer, 2005; Loy et al., 2005; Wilson and Reeder, 2005; Nicolas et al., 2017b).

The descriptions of the majority of the above *Talpa* species are based primarily on morphometry (Corti et al., 1985; Corti and Loy, 1987; Loy et al., 1993; Kryštufek, 1994; Rohlf et al., 1996; Loy and Capanna, 1998; Kryštufek and Benda, 2002; Kryštufek et al.,

2018; Selçuk et al., 2018; Sansalone et al., 2019) and dental traits (Nicolas et al., 2017a; Kryštufek et al., 2018). However, the highly conservative morphology of the genus, resulting from the functional constraints associated with fossoriality, have led to inconsistent morphological recognition of taxa that have confused taxonomic assignments in the past (Kryštufek and Vohralík, 2001; Bannikova et al., 2015; Kryštufek et al., 2018). Cytogenetic studies have also shown that the karyotypes of *Talpa* species are quite stable, with a diploid chromosome number (2n) of 34, and 62–64 autosomal arms (NFa) (Meylan, 1966; Dzuev et al., 1972; Todorovic´ et al., 1972; Capanna, 1981; Jimenez et al., 1984; Gornung et al., 2008; Sözen et al., 2012) throughout the genus, with the exception of *T. caeca* (2n = 36, NFa = 64 ) and *T. caucasica* (2n = 38, NFa = 62). Additionally it has been shown that *Talpa* species with almost identical karyotypes (*T. europaea* and *T. romana*) differ only slightly in localisation of 5S rRNA genes (Gornung et al., 2008), further suggesting taxa are not easily distinguished on cytogenetic features.

In contrast to this morphological and karyotypic homogeneity, molecular genetic techniques have been highly successful in *Talpa* taxonomy - highlighting the existence of morphologically cryptic, but genetically divergent lineages, which appear to constitute species. Bannikova et al. (2015) recently separated three such well-defined lineages in the Caucasus and Anatolia: *T. talyschensis* Vereschagin, 1945, *T. ognevi* Stroganov, 1948, and *Talpa levantis* sensu lato (s.l.). The first of these taxa was formerly considered a junior synonym of *T. levantis*; the second a junior synonym of *T. caucasica* (Hutterer, 2005). These findings highlight the fact that lineage diversity within the genus *Talpa* likely remains under-estimated, particularly in areas known to habour high biodiversity, and which likely served as refugia during Pleistocene glaciations. Turkey is likely to be one such area, since it not only hosts five out of the eleven currently known *Talpa* species (*T. caucasica, T. davidiana, T. europaea, T. levantis,* and *T. martinorum* – Kryštufek and Vohralík, 2009; Kryštufek et al., 2018; Selçuk et al., 2018) but it is also the region in which a number of other recent small mammal species/lineages originated (Gündüz et al., 2007). More generally, it is an important biodiversity hotspot in the West Palaearctic due to its complex topography, climatic conditions, and tectonic history (Myers et al., 2000). *Talpa* are strictly subterranean animals, with limited dispersal capability (Steinberg and Patton, 2000) and a high degree of territoriality (Ognev, 1928; Stein, 1950; Godfrey, 1957; Loy et al., 1994), and their diversification is likely to have been strongly impacted by such factors.

The Levant mole was first described from the vicinity of Trabzon (Maçka, Altındere, north-eastern Turkey) as a subspecies of the European *T. caeca* Savi, 1822 – *Talpa caeca levantis* Thomas, 1906. After being considered as subspecies of *T. caeca* for a period of time (Spitzenberger and Steiner, 1962; Osborn, 1964; Grulich, 1972), it was elevated to species rank by Spitzenberger (in Felten et al., 1973) based on external measurements and craniometrical variables, especially the relationships between condylobasal length and rostral breadth. Subsequent studies showed that the two species are clearly distinguished on diploid karyotypes (2n = 36 in *T. caeca* vs. 2n = 34 in *T. levantis*) and skull morphology (Zima and Král, 1984; Kryštufek, 1994; Selçuk and Kefelioğlu, 2017). Bannikova et al. (2015) showed that these two taxa are not particularly closely related, estimating that they diverged as early as the Late–Middle Pliocene, approximately 2.68 Myr, based on four concatenated nuclear genes. The range of the *T. levantis* s.l. extends west–east across the Anatolian Black Sea region, from South-eastern Bulgaria and Turkish Thrace as far as the southwestern coast of the Caspian Sea (Osborn, 1964; Doğramaci, 1989; Sokolov and Tembotov, 1989; Vohralík, 1991, Kryštufek, 2001; Popov and Miltchev, 2001; Bannikova et al., 2015). As such, it is the most common and widespread mole in Turkey, and inhabits various habitats from sea level to ca. 2000 m, in areas with rainfall ranging from 1000 to 2500 mm/year (Doğramaci, 1989; Kryštufek, 2001; Popov and Miltchev, 2001; this study). Bannikova et al. (2015) found relatively large cyt-*b* divergences (7%) between two sublineages within Anatolian and Caucasian *T. levantis* s.l., suggesting that this taxon, as currently defined, constitutes a pair of cryptic species. Of these sublineages, one occupies the majority of the Turkish range of *T. levantis* s. l., throughout the Black Sea coast, westwards to the Marmara region in Anatolia, whilst the other is found in Transcaucasia, and the adjacent parts of northeastern Anatolia. Bannikova et al., (2015) recognized that these two sublineages likely constitute separate species, but refrained from naming them since they lacked genetic data for moles from or close to the type locality of *T. levantis*, and so could not determine whether this name applied to the eastern or western sublineage.

Here we use a combination of nuclear and mitochondrial DNA sequence data to revisit the systematics and phylogeography of *T. levantis* s. l., including material from the type locality, in an attempt to stabilize the taxonomy of these moles and better understand their evolutionary history and population expansion.

## Material and methods

*Specimen collection*

Levant moles were collected from thirteen sites in northern Anatolia between 2007 and 2018 (Fig. 1), table 1 summarizes collection details. Standard voucher specimens (skins, skulls and various tissues in ethanol) are deposited in the Department of Biology, Faculty of Sciences and Arts, Ondokuz Mayıs University (OMUS), Samsun, Turkey for long term storage.

*Molecular analyses*

*DNA Extraction*

Total genomic DNA was extracted using Qiagen DNeasy tissue kits (QIAGEN Inc.) from tail tips, kidneys or liver preserved in 95% ethanol. Extracted DNA was suspended in nuclease-free water and DNA concentration quantified using a Nano-Drop spectrophotometer (NanoDrop Technologies, Wilmington, DE), adjusted to 25–50 ng/µL, and stored at -20 °C until used for PCR.

*PCR amplification and sequencing of the cyt-b gene*

A 1754 bp fragment encompassing a small part of tRNA-Glu (18 bp), the whole of the cyt-*b* gene (1140 bp), tRNA-Thr, tRNA-Pro (277 bp) and the 5' end (the hypervariable region I) of the D-loop (554 bp, excluding indels), spanning positions 14 163 to 15 913 of the *T. europaea* mitochondrial genome (GenBank Y19192, Mouchaty et al., 2000) was amplified with primers L14162 5'-GACATGAAAAATCATCGTTG-3' (modified from L14727-SP in Jaarola and Searle, 2002) and H15917 5'-CCTGAAGTAAGAACCAGATG-3' (modified from H16498 in Meyer et al., 1990). PCR amplifications were carried out in an S1000 thermal cycler (BIORAD) using Platinium *Taq* DNA polymerase (INVITROGEN). The PCR protocol consisted of an initial 2 min denaturation step at 95 °C, 35 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 45 s and extension at 72 °C for 1.5 min, and a final 7-min extension step at 72 °C. PCR products were purified using QIAquick kits (QIAGEN). Negative controls were included in all PCRs to check for contamination. After amplification, an aliquot was taken from each PCR reaction mix, and the amplified DNA fragment quantified in agarose gel by comparison with known quantities of phage λ DNA.

The complete cyt-*b* gene was sequenced in both directions using one of the amplification primers (L14162) plus three internal primers (H15351 5'-TCTCCATTGCTGGTTTACAAGAC-3', modified from H15915 in Irwin et al., 1991 and two

newly designed Levant mole specific primers, L14711 5'-GGTAGACAAAGCCACACTCAC -3' and H14935 5'-GAATGTAGTTGTCTGGATCTCC-3'). The position of the 3' end oligonucleotide of each primer is given relative to the published sequence of the common mole mtDNA (Mouchaty et al., 2000). Cycle sequencing reactions were carried out using BigDye Terminator cycle sequencing kits (Applied Biosystems). Amplifications and sequencing reactions were performed in an S1000 thermal cycler. Sequencing products were purified using DyeEx 2.0 Spin Kits (QIAGEN) and run in an ABI 3100 automated DNA sequencer (Applied Biosystems).

*Nuclear mitochondrial pseudogenes (numts) detection*

It is crucial to avoid confusion of true mitochondrial sequences with copies in the nucleus (pseudogenes – Mirol et al., 2000; Bensasson et al., 2001; Dubey et al., 2009). To screen for pseudogenes, three different PCRs, producing overlapping fragments, were performed on five randomly selected individuals from distinct localities. First, partial tRNA-Glu (42 bp), the entire cyt-*b* (1140 bp) and partial tRNA-Thr (26 bp) were amplified with primers L14138 5'-CCCACATGGAATTTAACCATGAC-3' (modified from Cb-M1 in Kurose et al., 2000) and H15351; second, partial tRNA-Glu (42 bp), the entire cyt-*b* (1140 bp), complete tRNA-Thr (69 bp), complete tRNA-Pro (70 bp) and the 5' end of the D-loop (554 bp) were amplified with primers L14138 and H15933; and third, part of tRNA-Glu (18 bp), the entire cyt-*b* gene (1140 bp), complete tRNA-Thr (69 bp), complete tRNA-Pro (70 bp) and the 5' end of the D-loop (554 bp) were amplified with primers L14162 and H15933. These three PCR products were then sequenced and aligned. Overlapping sequences were examined for any ambiguous bases, stop codons or open reading frame shifts, which might have indicated the presence of nuclear copies.

*PCR amplification and sequencing of breast cancer type 2 susceptibility protein (BRCA2) gene*

Amplification of a 927 bp portion of exon 11 of *BRCA2* was performed using the primer pair F1140a and R2050 (Bannikova et al., 2015). PCR conditions consisted of initial denaturing at 94 °C for 2 min; 30 cycles of 94 °C for 45 s, 64 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 6 min. PCR primers were used for sequencing in both directions.

*Sequence inspection and alignment*

All sequence traces were checked, aligned and ambiguous bases resolved by eye using Sequencher v4.5 (Gene Codes Corp.). Nucleotide and amino acid composition were analyzed using MacClade v4.08 (Maddison and Maddison, 2000).

*Phylogenetic analyses*

Nucleotide composition was analyzed and the frequency of each haplotype estimated using MacClade v4.08. To evaluate the degree of differentiation between sublineages of *T. levantis* s.l. compared to other species of the genus *Talpa*, and revisit the phylogenetic position of these, we also downloaded 78 cyt-*b* sequences from GenBank, from fourteen *Talpa* taxa (*T. altaica, T. aquitania, T. caeca, T. caucasica, T. davidiana, T. europaea, T. levantis* 'eastern' *T. levantis* 'western' (sensu Bannikova et al., (2015)), *T. martinorum, T. occidentalis, T. ognevi, T. romana, T. stankovici* and *T. talyschensis* – see Appendix). Phylogenetic relationships amongst cyt-*b* haplotypes were inferred with Maximum Parsimony (MP) and Maximum Likelihood (ML) algorithms implemented in PAUP v4.10b (Swofford, 2002) as well as Bayesian inference of phylogeny (BI) as implemented in MRBAYES v3.1.2 (Ronquist and Huelsenbeck, 2003). The Akaike information criterion (AIC) implemented in jMODELTEST v1.0 (Posada, 2008) was used to establish the most appropriate model of DNA substitution for our data, and this then employed in ML and BI analyses. The parsimony analyses were replicated 10 times with the heuristic search approach using the TBR swapping algorithm, steepest descent option and 10 random repetitions. Strict and 50% majority rule consensus trees were constructed from equally parsimonious MP trees. Bootstrap analysis of the MP tree was conducted with 1000 replications using 10 random repetitions of each replication. ML analysis was conducted using the heuristic search, the 'as is' addition replicate. Branch support was assessed using 1000 non-parametric bootstrap replicates. BI analysis involved four Markov Chains of 20 million generations, with trees being sampled every 100 generations and a burn-in of 25%. The software tool TRACER v1.6 (Rambaut et al., 2014) was used to observe the parameters and to determine the number of trees needed to reach stationarity (burn-in). After discarding burn-in trees and evaluating convergence, remaining samples were retained for generating 50% majority rule consensus tree and calculating posterior probabilities. Based on the existing supported phylogenetic hypothesis (He et al., 2014), cyt-*b* sequences of four eastern Asian mole species of three genera (*Mogera robusta* Nehring, 1891, *Euroscaptor mizura* (Günther, 1880), *Euroscaptor longirostris* (Milne-Edwards, 1870) and *Parascaptor leucura* (Blyth, 1850)) were used as outgroups in phylogenetic analyses (see Appendix).

Relationships amongst cyt-*b* sequences of *T. levantis* s.l. were also investigated by constructing a network using the median-joining (MJ) algorithm implemented in the software NETWORK v4.6.1.2 (Bandelt et al., 1999; http://www.fluxus-engineering.com). We included all newly sequenced specimens (24 individuals) and all specimens of *T. levantis* 'western' (seven individuals) and *T. levantis* 'eastern' (five individuals) available in GenBank (Table 1).

As some of the *BRCA2* sequences in GenBank were shorter than ours or contained some unresolved positions, the *BRCA2* alignment was limited to 656 bp to match newly obtained haplotypes with published data, leading to some redundant sequences and some loss of phylogenetic signal. Phylogenetic relationships between *BRCA2* sequences were also investigated using MP (1000 bootstrap replicates), ML (1000 bootstrap replicates) and BI approaches using the settings described above. The nucleotide substitution model selected according to the AIC by jMODELTEST was employed in ML and BI analyses. We included all newly described haplotypes (Hap.1-9) and those of *Talpa* species available in GenBank (Table 1, Appendix). Unfortunately, two recently described species (*T. aquitania* and *T. martinorum*) could not be included in the analyses since there are no *BRCA2* sequences available. The full dataset contained 31 *Talpa* haplotypes. Again, *BRCA2* sequences of the same four eastern Asian mole species were used as an outgroup in the phylogenetic analysis (Appendix).

*Molecular diversity*

Genetic diversity estimates were calculated for cyt-*b* sublineage of interest (western). Nucleotide diversity ($\pi$) and haplotype diversity ($H_d$) were calculated using ARLEQUIN v3.5 (Excoffier and Lischer, 2010). Another measure of nucleotide diversity, $\theta w$ (computed from the number of segregating sites; Watterson, 1975), was calculated using DNASP v5 (Librado and Rozas, 2009). DNA net and mean divergences (*Da* and *D*xy; Nei, 1987) between sublineages were estimated under the Kimura 2-parameter (K2P; Kimura, 1980) model using MEGA X (Kumar et al,. 2018).

*Divergence time estimation and population demographics*

We obtained an approximate estimation of the divergence time between *T. levantis* sublineages using the formula $T = Da/2\mu$, where $2\mu$ is the divergence rate, using a substitution rate for the cyt-*b* gene of 0.01407 changes per site per lineage per million years (Colangelo et

al., 2010). We performed a series of statistical tests to test the hypothesis of sudden population expansion for the two sublineages. First, mismatch distributions (Rogers and Harpending, 1992; Rogers, 1995) were calculated for each sublineage to examine historical changes in population size with ARLEQUIN. To compare observed data with those expected under a sudden expansion model we conducted goodness-of-fit tests based on the sum of squared deviations (SSD) and Harpending's raggedness index (*rg*) (Harpending, 1994; Schneider and Excoffier, 1999) using 10 000 parametric bootstrap replicates.

The mismatch distribution of pairwise genetic differences was also used to calculate expansion times (Rogers and Harpending, 1992). The change of effective female population size (*N*) since population expansion is used to estimate the time since expansion in generations. A population at equilibrium with $N_0$ changes to $N_1$ at $\tau$ units of mutational time. The modal value $\tau$ is determined from the distribution of pairwise genetic differences in the extant population. The parameters of the model are given by: $\theta_0 = 2N_0u$, $\theta_1 = 2N_1u$ and $\tau = 2ut$. Here, *t* is the time since expansion in generations and *u* is the mutation rate of the entire DNA fragment (expressed as $u = 2\mu k$, where μ is the mutation rate per nucleotide per generation and *k* is the length of the sequence). Population expansion times were estimated assuming a mean clock rate of $1.407 \times 10^{-8}$ substitution/site/year, as observed in the genus *Talpa* (Colangelo et al., 2010). Second, we conducted tests of neutral equilibrium assumptions using two widely used statistics, Tajima's *D* (Tajima, 1989) and Fu's $F_S$ (Fu, 1997), as additional assessments of possible population expansion. Tajima's *D* statistic tests the null hypothesis that the average number of pairwise nucleotide differences and number of segregating sites in the sample are equal (Tajima, 1989). This method explicitly tests for selective neutrality and is based on an infinite-sites model assuming no recombination. Fu's *Fs* statistic is also a selective neutrality test based on the infinite-sites model, assumes no recombination and tests the probability of having no fewer haplotypes than the observed number in the sample (Fu, 1997). Negative values for these two statistics are most often attributed to positive selective sweeps, population size expansion or background selection. Used in combination, the tests can provide evidence for or against particular evolutionary mechanisms. As population size expansion leads to changes in the frequency distribution of haplotypes (i.e. an excess of haplotypes or an excess of singleton mutations), we also calculated Strobeck's *S* statistic (Strobeck, 1987) and $R_2$ (Ramos-Onsins and Rozas, 2002). Strobeck's *S* compares the observed number of haplotypes to that expected based on the

frequency distribution derived from the inferred mutation rate, whereas $R_2$ is based on the difference between the number of singleton mutations and the average number of nucleotide differences, where lower $R_2$ values are expected under population expansion. The $R_2$ test is very powerful in detecting population expansions in small sample sizes (Ramos-Onsins and Rozas, 2002). Significance values for Tajima's *D*, Fu's $F_S$ and $R_2$ were obtained from 10 000 coalescent simulations conditioned on theta as implemented in DNASP. The cut-off level for statistical significance was 0.05. For Fu's $F_S$, significance at the 0.05 level was indicated when *P* values were < 0.02 (Excoffier and Lischer, 2010).

## Results

### *Sequence analysis and phylogenetic reconstructions*

#### *Mitochondrial DNA*

The entire cyt-*b* gene (1140 bp) was sequenced from 24 Levant moles from 13 localities, revealing 15 unique haplotypes (Hap.1-15) with K2P-distance values 0.09-3.34% (overall mean K2P distance of 1.54%). Eleven of these haplotypes were found in one individual, four in more than one animal (Table 1). No internal stop codons or insertion/deletions were detected. Sequences with the same properties were also obtained using the alternative primer pairs for amplification and sequencing, making it reasonable to assume that no mitochondrial nuclear mitochondrial insertions (numts) were sequenced. The full dataset, including novel and published *Talpa* sequences contained 425 variable sites, of which 381 were parsimony informative, with 293 synonymous and 32 non-synonymous changes across all taxa. The substitution model supported was the General Time Reversible, with specified substitution types (AC-1.0052, AG-31.6171, AT-0.7223, CG-0.1456, CT-18.9234, GT-1.0), proportion of invariable sites (0.5570), gamma shape parameter (1.1580) and nucleotide frequencies (A-0.3572, C- 0.3041, G-0.0941, T-0.2446).

The phylogenetic hypothesis for species of *Talpa* based on cyt-*b* gene sequences from ML is shown in Fig. 2. The different phylogenetic methods used (BI, ML and MP) all produced similar topologies. Our tree broadly agrees with previous phylogenetic studies of the genus (Colangelo et al., 2010; Bannikova et al., 2015; Kryštufek et al., 2018), including the separation of Levant moles into an eastern and western sublineage. Specimens from the type locality of *T. levantis* (possessing Haplotype 1), fall into the western sublineage (see Fig. 2). The network profile of 26 haplotypes of *T. levantis* is shown in Fig. 3A. This haplotype

network was consistent with the results of the phylogenetic analyses, providing an enhanced visualization of intraspecific genetic variation, with many substitutions accumulated between the western and eastern sublineages of *T. levantis* s.l. The two sublineages were connected via two median vectors, separated by 45 mutational steps. There were no shared haplotypes between the two sublineages, suggesting complete lineage sorting. The majority of the haplotypes in the western (Hap.5-15, KP717339, KP717340 and FN640572) sublineage belonged to one group with short to long branches between the haplotypes, connected via median vectors. Three smaller group of haplotypes formed more distant sub-clusters within the western sublineage (Hap.1-3; FN640571; KP717336 and KP717338), suggesting additional subdivisions within this sublineage. One sub-cluster, comprising haplotypes Hap.1-3 is restricted to north-east Anatolia, including the locality of topotypes of *T. levantis* (localities 5 and 6), another sub-cluster comprising haplotypes FN640571, KP717336 and KP717338 is restricted to north-west Anatolia (localities 21-23), whilst the sub-cluster comprising the remaining 18 haplotypes is distributed across north-central Anatolia (localities 7-20) (Fig. 1, Table 1). The eastern sublineage is represented by fewer sequences than the western, but it is nevertheless clear that the haplotype (FN640570) from Çam geçidi, Ardahan (locality 4) in northeastern Anatolia is rather strongly differentiated from the four relatively closely related haplotypes (KP717334, KP717335, KP717337 and FN640574) from Russia (Nalchik, locality 1) and Armenia (Fioletovo and Margahovit, localities 2 and 3).

*Nuclear DNA*

Sequences of a 927 bp portion of exon 11of *BRCA2* were generated from 24 moles from 14 localities, revealing nine unique haplotypes (Hap.1-9), six of which were found in one individual, three in more than one (Table 1). When combined with available (shorter – see Materials and methods) published *Talpa BRCA2* sequences, the dataset contained 31 *Talpa* haplotypes of 656 bp long (see Appendix). This alignment contained 43 variable sites, 22 of which were parsimony informative. jMODELTEST supported the Hasegawa-Kishino-Yano (HKY) substitution model for these data, maximum likelihood analysis resulting in an optimal which was only partly resolved due to the relatively low number of polymorphic sites (Fig. 4). BI and MP analyses both produced topologies similar to that of ML. As with the cyt-*b* phylogeny, the *BRCA2* data separated *T. levantis* s.l. into a geographically widespread western sublineage represented by nine haplotypes (Hap.1-9, including Hap.1 from the topotypes of *T. levantis*, locality 5 in Fig. 1) from this study, plus KP717122 from Uzungüney

Köyü, Zonguldak (locality 25 in Fig. 1), and a more restricted eastern sublineage represented here by one haplotype only, KP717115 from the Nalchik region in Russia (locality 1 in Fig. 1).

*Diversity, divergence time and demographic history*

The western sublineage of *T. levantis* contained 21 cyt-*b* haplotypes (15 new in the present study, six additional haplotypes from GenBank), resulting in a haplotype diversity ($H_d$) of 0.996, nucleotide diversity ($\pi$) of 1.894% and a $\theta w$ of 2.447%. Mean and net divergence estimates between cyt-*b* sequences in eastern and western sublineages of *T. levantis* were 7.28% and 5.75%, respectively. This net divergence estimate equates to a separation time of ~1.91 Mya, suggesting that the two sublineages diverged in the early Pleistocene. For the western sublineage of *T. levantis*, neutrality tests for cyt-*b* data revealed no significant deviation from neutrality and the expected equilibrium. Both Tajima's *D* (*D* = –0.9346, *P* = 0.1765) and Fu's *Fs* (*Fs* = –0.8062, *P* = 0.3982) yielded negative but nonsignificant values. Strobeck's *S* was low (0.816) and Ramos-Onsins and Rozas' $R_2$ test was not significant ($R_2$ = 0.0894, *P* = 0.1816). These results were corroborated by a mismatch distribution that showed a wave signal (multimodal distribution) consistent with constant population size or demographic equilibrium (Fig. 3B). Conversely, the goodness of fit test was consistent with a hypothesis of demographical expansion (SSD = 0.0239, *P* = 0.1241; *rg* = 0.0165, *P* = 0.1812). Demographic analyses were not possible for the eastern sublineage due to low sample size (n = 5).

## Discussion

Our data confirm that *Talpa levantis* s.l. is divisible into distinct eastern and western sublineages, which apparently diverged from each other in the early Pleistocene. Given the degree of DNA sequence divergence observed between sublineages, and the fact that these remain robust and geographically coherent in light of our more extensive sampling, we agree with Bannikova et al. (2015) that these should be considered as separate species. Mean cyt-*b* K2P divergences observed between most mole species examined previously range from 8.6% (*T. europaea* vs. *T. occidentalis*) to 15.6% (*T. altaica* vs. *T. romana*) (Colangelo et al., 2010; Feuda et al., 2015). However, slightly lower genetic divergence (7.7%) was observed between *T. occidentalis* and its recently described relative *T. aquitana* (Nicolas et al., 2017a,b). The 7.28% cyt-*b* divergence between sublineages revealed here is very close to this value.

The inclusion of *T. levantis* specimens from the type locality allows us to fix the nomenclature of Levant moles for the first time. Topotypical *T. levantis* belong to the western sublineage, on both mitochondrial and nuclear data and it is therefore this taxon which is the

true *T. levantis* sensu Thomas, 1906. As discussed by Bannikova et al. (2015) this therefore means that the oldest name available for the eastern clade is *T. transcaucasica* Dahl, 1944. Traditional delineation of species-level entities in morphologically constrained subterranean mammals such as moles is intrinsically difficult (see Nevo, 1979), and there are, to date, no known diagnostic morphological characters on which *T. levantis* and *T. transcaucasica* can be separated. Selçuk et al. (2018) examined skull and mandible morphometrics of selected Turkish mole species, including specimens of *T. levantis* s.l. from both eastern and western areas; i.e. both *T. levantis* s. str. and *T. transcaucasica* as defined here. Their results reveal relatively high morphological variability in *T. levantis* s.l. compared to the other taxa studied, suggesting that morphometric separation between *T. levantis* and *T. transcaucasica* may be possible. Future studies exploring this, together with additional nuclear markers, would clearly be useful to confirm our taxonomic hypothesis. There is no clear geographical barrier currently separating *T. levantis* s. str. and *T. transcaucasica*, although the species appear to occur either side of the Anatolian Diagonal (sensu Davis, 1971), a biogeographical break between central and eastern Anatolia which is observed in a wide variety of organisms (Çıplak, 2003; Gündüz et al., 2007; Mutun, 2010; Kapli et al., 2013; Stümpel et al., 2016; van Riemsdijk et al., 2017). As discussed by Gür (2016), this seems to result largely from differences in temperature seasonality, which apparently also existed during the Last Glacial Maximum and possibly earlier. The identity of *T. levantis* s.l. reported from southeastern Bulgaria and Turkish Thrace remains unclear, due to the absence of molecular data from these regions. A number of small mammal species are known to span the Bosphorus (e.g. Dubey et al., 2007, Helvacı et al., 2012) and it is possible that European populations are indeed conspecific with Anatolian ones. Alternatively, what has been considered as *T. levantis* in Europe may belong to yet another distinct taxon, something which should be tested in the future. It is clear, however, that Turkey is a very important centre for mole speciation and diversity, now being home to six distinct species, more than any other comparable geographical region.

Compared with other mole species, *T. levantis* s. str. has relatively high haplotype and nucleotide diversity ($H_d$ = 0.996, $\pi$ = 1.894%). Based on cyt-*b* data, Canestrelli et al. (2010) estimated haplotype and nucleotide diversities for the six haplogroups of *T. romana* to be 0.81-0.98 and 0.18-0.77%, respectively. Nicolas et al. (2017b) found almost equally high levels of haplotype diversity but relatively low nucleotide diversity values for three mole species: $H_d$ = 0.932, $\pi$ = 0.371% in *T. europaea*; $H_d$ = 0.908, $\pi$ = 0.830% in *T. aquitania* and

$H_d$ = 0.892, $\pi$ = 0.724% in *T. occidentalis*. The high diversity values observed in *T. levantis* s. str. suggest either the maintenance of relatively large populations throughout the species evolutionary history, and/or its survival in a number of separate refugia during the Pleistocene (Grill et al., 2009; Vega et al., 2010; Nicolas et al., 2017b). Our understanding of Pleistocene palaeoenvironmental history in northern Anatolia remains incomplete, but it is clear that the region hosts a large number of microendemics, in a variety of plant and animal taxa (e.g. Davis, 1971; Wielstra et al., 2010; Trizzino et al., 2013), which strongly suggests the occurrence of multiple, long-term refugial areas in the region, as has been hypothesised elsewhere (e.g. Lindell et al., 2006; Soltis et al., 2006; Byrne et al., 2008; Canestrelli and Nascetti, 2008; Ursenbacher et al., 2008; Gonçalves et al., 2009; Wang et al., 2009; Vega et al., 2010; Bidegaray-Batista et al., 2016; van Riemsdijk et al., 2017; Wielstra et al., 2017). Genetic substructuring and mutation rate heterogeneity may produce a multimodal pattern, even in demographically unstable populations (Marjoram and Donnelly, 1994; Aris-Brosou and Excoffier, 1996). The multimodality of the mismatch distribution of *T. levantis* s. str. seems to result from the presence of different haplogroups, rather than demographical stability. This interpretation is supported by the phylogenetic and network analyses of cyt-*b* data, which suggest three distinct sub-clusters within this taxon (see Fig. 2 and 3A), perhaps corresponding to populations which have persisted in separate refugial areas which retained suitable habitat during much of the species evolutionary history. If we assume a hypothesis involving some degree of population growth for *T. levantis* s. str., demographic expansion for this taxon, based on our estimated values of $\tau$, occurred approximately 296,000 years BP in the late Pleistocene.

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872

873 **Figure legends**

874 **Figure 1.** Sampling locations and habitat of *T. levantis* s.l. in Anatolia. **A)** Map of sampling
875 locations (see Table 1). ● *T. levantis* s.l. 'western', this study; ○ *T. levantis* s.l. 'western';
876 previous studies and ▲ *T. levantis* s.l. 'eastern', previous studies. Western and Eastern
877 sublineages follow Bannikova et al. (2015). **B)** Typical habitat of *T. levantis* s.l. at the type
878 locality (Altındere, locality 5); 1650 m a.s.l., Trabzon, Anatolia. Photograph S. Demirtaş.
879

880 **Figure 2.** Results of BI, ML and MP analyses combined on a ML tree based on cyt-*b* sequences
881 of *Talpa* and outgroup species. Numbers at nodes indicate posterior probabilities (BI) and
882 bootstrap support values (ML and MP) which are reported only for the key nodes. Bayesian
883 posterior probabilities (≥0.90) and bootstrap supports (≥70%) are shown. The locality numbers
884 for the haplotypes of *'western'* and *'eastern'* sublineages are given in parentheses just after the
885 haplotype IDs according to the numbering on map on the Figure 1 and Table 1. For the
886 geographical origins of the published sequences see Table 1 and Appendix.

887

888 **Figure 3. A**) Median-joining network constructed using cyt-*b* sequences of *Talpa levantis* s.l.
889 The size of each circle is proportional to the frequency of the particular haplotype in the sample.
890 Median vectors are indicated by blank circles. For the geographical origins of the published
891 sequences see Appendix. **B**) Mismatch distribution of cyt-*b* sequences for the western
892 sublineage of *Talpa levantis* s.l., showing observed and expected values (see text).

893

894 **Figure 4.** Results of BI, ML and MP analyses combined on a ML tree based on *BRCA2*
895 sequences of *Talpa* and outgroup species. Numbers at nodes indicate posterior probabilities
896 (BI) and bootstrap support values (ML and MP). Bayesian posterior probabilities (≥ 0.90) and
897 bootstrap supports (≥70%) are shown. The locality numbers for the haplotypes of '*western*' and
898 '*eastern*' sublineages are given in parentheses just after the haplotype IDs according to the
899 numbering on map on the Figure 1 and Table 1. For the geographical origins of the published
900 sequences see Table 1 and Appendix.

**Table 1.** Populations of *T. levantis* s.l. sampled in this and previous studies (Colangelo et al., 2010; Bannikova et al., 2015), with details on the distribution of haplotypes (if more than one haplotype is present in a population the frequency is stated in parentheses). Cyt-*b* haplotypes (Hap.1-15, GenBank accession numbers: XXXXXXXX–XXXXXXXX) and *BRCA2* haplotypes (Hap.1-9, GenBank accession numbers: YYYYYYYY–YYYYYYYY) were new to this study. Locality codes correspond to Fig. 1. See Appendix for sequences from other taxa used in phylogenetic analyses.

| Locality | Locality code | Latitude/longitude | *n* | GenBank Accession No/Haplotype ID | | Reference |
|---|---|---|---|---|---|---|
| | | | | Cyt-*b* | *BRCA2* | |
| RUSSIA | | | | | | |
| Nalchik | 1 | 43º46' N, 43º66' E | 2 | KP717334 | KP717115 | Bannikova et al., 2015 |
| | | | | FN640574 | | Colangelo et al., 2010 |
| ARMENIA | | | | | | |
| Fioletovo | 2 | 40º71' N, 44º71' E | 1 | KP717337 | | Bannikova et al., 2015 |
| Margahovit | 3 | 40º74' N, 44º70' E | 1 | KP717335 | | Bannikova et al., 2015 |
| TURKEY | | | | | | |
| Çam geçidi, ARDAHAN | 4 | 41º20' N, 42º55' E | 1 | FN640570 | | Colangelo et al., 2010 |
| Taşköprü, Altındere, TRABZON | 5 | 40º64' N, 39º67' E | 2 | Hap.1 | Hap.1 | |
| Görele, GİRESUN | 6 | 41º02' N, 39º02' E | 2 | Hap.2 | Hap.1 | |
| | | | | Hap.3 | | |
| Batlama Deresi, GİRESUN | 7 | 40º88' N, 38º34' E | 1 | Hap.4 | Hap.2 | |
| Ulubey, ORDU | 8 | 40º85' N, 37º76' E | 2 | Hap.5 | Hap.3 | |
| | | | | Hap.6 | Hap.4 | |
| Geyikçeli Köyü, Fatsa, ORDU | 9 | 40º93' N, 37º37' E | 1 | Hap.7 | Hap.5 | |
| Kurupelit, SAMSUN | 10 | 41º37' N, 36º19' E | 3 | Hap.8 | Hap.6 (2) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | Hap.7 | |
| İncesu Köyü, SAMSUN | 11 | 41º38' N, 36º18' E | 1 | Hap.9 | Hap.8 | |
| Erikli Köyü, SAMSUN | 12 | 41º37' N, 36º05' E | 1 | Hap.10 | Hap.6 | |
| Karakavuk, SAMSUN | 13 | 41º38' N, 36º11' E | 1 | Hap.10 | Hap.6 | |
| Kürtler, SAMSUN | 14 | 41º48' N, 36º09' E | 1 | FN640572 | | Colangelo et al., 2010 |
| Dereköy, Ondokuzmayıs, SAMSUN | 15 | 41º45' N, 36º11' E | 4 | Hap.11 | Hap.3 (2) | |
| | | | | Hap.12 | | |
| | | | | Hap.13 (2) | Hap.6 (2) | |
| Kızılırmak deltası, Bafra, SAMSUN | 16 | 41º67' N, 35º98' E | 4 | Hap.12 (2) | Hap.6 (3) | |
| | | | | Hap.13 | Hap.1 | |
| | | | | Hap.14 | | |
| Uluağaç Köyü, Bafra, SAMSUN | 17 | 41º26' N, 35º58' E | 1 | Hap.13 | Hap.3 | |
| Abalı Köyü, SİNOP | 18 | 42º02' N, 34º98' E | 1 | Hap.15 | Hap.9 | |
| Sefercik, Filyos, ZONGULDAK | 19 | 41º55' N, 32º03' E | 2 | KP717339 | | Bannikova et al., 2015 |
| | | | | KP717340 | | Bannikova et al., 2015 |
| Çaycuma, ZONGULDAK | 20 | 41º43' N, 32º15' E | 1 | KP717339 | | Bannikova et al., 2015 |
| Uzungüney Koyu, ZONGULDAK | 21 | 41º37' N, 31º70' E | 1 | KP717338 | KP717122 | Bannikova et al., 2015 |
| Alapli, ZONGULDAK | 22 | 41º18' N, 31º40' E | 1 | KP717336 | | Bannikova et al., 2015 |
| Uludağ, BURSA | 23 | 40º10' N, 29º24' E | 1 | FN640571 | | Colangelo et al., 2010 |